PHYSICAL AND TEMPORAL FACTORS INVOLVED IN THE DEATH OF YEAST AT SUBZERO TEMPERATURES

PETER MAZUR

From the Department of Biology, Princeton University, Princeton, and the Biology Division, Oak Ridge National Laboratory, Oak Ridge. Present address: Biology Division, Oak Ridge National Laboratory, Oak Ridge.

ABSTRACT The survival of yeast cells after exposure to subzero temperatures was affected by the cooling and warming velocity, temperature itself, and the physical state of the water surrounding the cells. The cells were injured only when the external medium was frozen and then only when the temperature was −10° or below. Survival dropped abruptly between −10° and −30° regardless of whether the cells were suspended in water or 0.1 M solutions of KH_2PO_4, NaCl, or $CaCl_2$. The critical temperature range of −10° to −30° was unrelated to the temperatures at which the suspending fluids completely solidified, these temperatures being −0.3°, −11°, −30°, and −71° for the four liquids, respectively. Survival at −30° or below was greatly affected by the rate at which the cells were cooled or warmed, with higher survivals being obtained with slow cooling and with rapid warming. Length of exposure at −30° was not a factor; injury was inflicted within 1 minute. The results are interpreted as indicating that death is a result of intracellular ice formation. Internal freezing is believed to occur when external ice crystals grow through aqueous channels in the cell wall or membrane and seed supercooled water in the cell interior.

INTRODUCTION

A decrease in the temperature of biological systems decreases the rate of most chemical and many physical processes and results ultimately in the freezing of water. The decreased reaction rates provide a useful method for extending the life of ordinarily short-lived substances ranging from free radicals (1) to living cells themselves (2). With living cells, however, this promising aspect of low temperatures is often negated by the concomitant freezing of water, with the result that most interest in freezing has been centered on preventing ice crystal formation or eliminating its lethal consequences. But freezing may also have its more positive aspects related to the possibility that the conversion of liquid water to solid could provide an experimental method for investigating the nature and role of liquid water in the normal cell.

Although the dual action of low temperatures in reducing reaction rates and inducing freezing seems rather straightforward, the responses of living cells to low temperatures have proved to be bewilderingly multifarious. The responses are complex because these two primary consequences give rise to a host of secondary consequences including changes in density and volume, solute concentration, locus and size of ice crystals, and locus of water molecules. Moreover, the existence and prominence of a particular phenomenon are affected not only by the characteristics of the specific cell under study but also by physical, chemical, and temporal factors such as cooling and warming velocities, temperature, duration of exposure, and type of solute molecule present.

This complex interplay of many factors made it imperative in this initial study to restrict the experimental variables to a minimum and it was decided to examine the effects of the physical-temporal factors just mentioned on the survival of the yeast *Saccharomyces cerevisiae*. Yeast was selected because (*a*) its viability can be determined relatively unambiguously by plate count assay, (*b*) it is large enough to permit cytological and morphological observations, and (*c*) a good deal is known of its biochemical make-up and activities, its physiological processes, and its genetics. Injury was found to be associated with high cooling rates, low warming rates, and temperatures below a certain critical range. It was almost instantaneous and required the presence of external ice crystals. These results are similar to those obtained previously with spores of the fungus *Aspergillus flavus*, (3-5) and cells of the bacterium *Pasteurella tularensis*, (6) a similarity that is rather surprising in view of the marked biological and physiological differences in the three cells.

MATERIALS AND METHODS

Cultures. The stock culture obtained through the kindness of Dr. L. J. Wickerham was a diploid strain of *Saccharomyces cerevisiae* (NRRL Y-2235 Diploid). Substocks were maintained on malt extract, yeast extract agar[1] slants at 4° and transferred monthly. Cells for individual experiments were obtained by transferring from the substocks to a slant of a predominantly synthetic agar medium,[2] which was incubated at 29° for 48 hours. Then 5 ml of sterile M/15 KH_2PO_4 was added to the slant, the yeast growth scraped off, and 0.2 ml of the resulting suspension transferred to 50 ml of liquid medium[2] in a 200 or 250 ml Erlenmeyer flask. The flask contents were agitated on a rotary shaker at 172 cycles/min. at 29-30° for 24 hours.

Preparation of Cell Suspensions for Low Temperature Exposure. One or two

[1] This formula was suggested by Dr. Wickerham: Yeast extract, 3 gm; malt extract, 3 gm; peptone, 5 gm; glucose, 10 gm; agar, 20 gm; distilled water, 1000 ml.

[2] The formula for both the solid and liquid media, obtained through the courtesy of Dr. W. Nickerson, Institute of Microbiology, Rutgers University, is as follows: Glucose, 20 gm (added separately in case of liquid medium); $(NH_4)_2SO_4$, 3.0 gm; KH_2PO_4, 3.0 gm; $MgSO_4 \cdot 7H_2O$, 0.25 gm; $CaCl_2 \cdot 2H_2O$, 0.25 gm; monosodium glutamate, 1.0 gm; yeast extract, 0.1 gm; distilled water to make 1000 ml. The formula for solid medium was the same with the addition of 20 gm of agar per liter.

10 ml aliquots of the Erlenmeyer culture were centrifuged at about 1000 *g* for 5 minutes, the supernatant was decanted, and the cells were washed twice by centrifugation and decantation with 10 to 12 ml volumes of sterile distilled water, and washed a third time with sterile deionized distilled water. Finally, sufficient sterile deionized distilled water was added to the packed cells to yield a total cell count of $\sim 2 \times 10^8$/ml (the cell count in the original shake culture was estimated by hemocytometer count and was 2 to 4×10^7/ml). When the final suspending medium was a salt solution, the cells were washed once in distilled water, and then washed twice and finally suspended in the desired solution. Reagent grade chemicals were used to make these solutions .

Freezing tubes were fashioned by fusing one end of 90 mm lengths of pyrex tubing, 6.90 to 7.00 mm O.D. Into each tube was pipetted, with a long-tipped measuring pipette, 0.100 ml of the cell suspension and the tubes were plugged with cotton.

Exposure to Low Temperature. One-gallon capacity cylindrical Dewar flasks containing 2 to 3 inches of ethanol served as cooling baths. A commercial low temperature bath[3] controlled by a speedomax H recorder[4] was also used in later experiments. To achieve high cooling rates, the freezing tubes were three-fourths immersed in ethanol precooled with dry ice to the desired temperature. The resultant cooling rate of the suspension depended on the bath temperature and was approximately 15°, 50°, and 300°/min. (measured from −5° to 5° above the final bath temperature) when the ethanol baths were at −15°, −30°, and −75°, respectively. In one experiment, suspensions were cooled at a consistent rate of 50°/min. to −30°, −45°, −60°, or −75° by successively transferring the freezing tubes to baths held at these temperatures. In other experiments, suspensions were cooled at 17°/min. to −15°, −20°, −25°, or −30° by successively transferring tubes stepwise to baths held at those temperatures. In both cases, the transfer was made when the temperature of the cell suspension dropped within 4° of the temperature of the bath. Other variations of this stepwise cooling were used and will be noted. All cooling rates produced by these techniques of immersing tubes in precooled baths will be referred to as rapid.

Slow cooling was achieved in the earlier experiments by placing the freezing tubes in a 3 liter beaker containing a 2 inch layer of ethanol at 10°. The temperature of the ethanol was then slowly dropped at about 1.2°/min. by manually adding small pieces of dry ice. In later experiments, the suspensions were slowly cooled by decreasing the setting of the Leeds and Northrup controller at 1°/min., thereby reducing the temperature of the ethanol in the canalco low temperature bath at that rate.

The water surrounding the cells either froze spontaneously at −9° to −16° or else was induced to freeze at higher temperatures by inserting the ice-filled tip of a sterile Pasteur pipette into the supercooled suspension and then immediately withdrawing it. The frozen solution in the tip was the same as that surrounding the cells and was at the same temperature as the latter at the moment of seeding.

After being cooled, the suspensions were held at the desired minimum temperature for times ranging from 10 to 120 minutes, the time being adjusted so that all suspensions in a given experiment spent the same total time below 0° regardless of the time required for cooling or warming. The suspensions were then warmed either rapidly or slowly. For rapid warming, the tubes were removed from the ethanol bath and plunged directly into water at 35°. The resulting rate of warming ranged from 1140°/min. from −70° to 0° to 500°/min. from −15° to 0°. When slow warming was desired, the tubes were re-

[3] Canal Industrial Corp., Bethesda.

[4] Leeds and Northrup Co., Philadelphia, 0 to 10 mv range.

moved from the ethanol bath and placed in a 1 liter beaker containing 300 to 325 ml ethanol previously cooled to the temperature of the bath. The ethanol in the beaker w then allowed to warm by contact with room temperature air. The rate of warming rang from 1°/min. from −75° to −2° to about 0.6°/min. from −10° to −2°. Warmi was continued until the cell suspensions had thawed and reached a temperature of +5

Temperatures were measured with 30 or 40 gauge copper-constantan thermocoupl attached to a Leeds and Northrup D.C. microvolt amplifier and recording millivolt pote tiometer or directly to thermocouple recorders (Leeds and Northrup speedomax and G).

Controls. Controls consisted of the usual "freezing" tubes containing 0.1 ı aliquots of suspensions of yeast in water or salt solutions held at room temperatu during the course of the experiment. Each control and experimental condition was repı sented by duplicate or, more usually,triplicate samples (freezing tubes).

Determination of Survival by Plate Count. The 0.1 ml of cell suspension in t freezing tubes was quantitatively transferred by syringes or Pasteur pipettes to dilutic bottles containing M/15 KH_2PO_4 as diluent. Samples were diluted to appropriate leve and 0.1 ml aliquots from one to three 10-fold dilution levels were spread on the surfa of duplicate or triplicate Nickerson yeast agar plates. Plates were incubated inverted 29° for 48 to 66 hours and colony counts made. The 66 hour incubation period was t more usual, and resulted in insignificantly higher colony counts than the shorter perio Percentage survival was calculated by dividing the average cell count per milliliter of t experimental material by the average cell count of uncooled controls. Average cell cou was obtained by dividing the total number of colonies on plates from all dilution lev giving countable results (1 to 600 colonies per plate) by the total amount plated cc rected for the dilution factor. The standard error ($s_{\bar{x}}$) was calculated from the me percentage survivals of each sample of the two or three subjected to a given treatmer

Methylene Blue Staining. To 1 part of a 10-fold dilution of the treated c suspension was added 1 part of a 1:10,000 dilution of methylene blue, U.S.P. (Nation Aniline and Chemical Co.) in distilled water. The suspension was thoroughly mixed a allowed to stand 30 minutes at room temperature before making differential cour under the microscope. When more than 1 hour elapsed between thawing of the suspe sion and adding methylene blue, the suspensions were held at 4° until 15 to 20 minut before the stain was to be added. Cells were tallied as either unstained (able to redu the dye) or stained (unable to reduce the dye). Cells in which the staining was so pale to be questionable were also classified as stained, but these ambiguous ones did not cor prise more than 1 or 2 per cent of the sample counted. At least 300 cells were counted each sample.

Electrical Resistance Measurements. The resistivity of several aqueous sol tions as a function of temperature was determined with a Leeds and Northrup 60 cyc conductivity bridge (model 4960) and dip type conductivity cells[5] with cell constan of 0.100 or 0.200. About 65 ml of the solution was introduced into a 200 ml capaci unsilvered cylindrical Dewar flask. Copper-constantan thermocouples were inserted the vicinity of the cell electrodes, and the cell was placed in the solution. The Dewar fla was then immersed in liquid nitrogen and allowed to cool until the resistivity rose 10^7 ohm-cm or above. It was then transferred to a room temperature water bath, whe it remained until the solution had reached the freezing point (−0.5° to 0°) and appr ciable melting was visible.

[5] Industrial Instruments Co., Cedar Grove, New Jersey.

RESULTS

Effect of Cooling Rate, Lowest Temperature of Exposure, and Warming Rate. As shown by the data in Table I, the survival of yeast was affected by the rate at which the cells were cooled, the lowest temperature to which they were cooled, and the rate at which they were warmed. In cells cooled to −10°, survivals were high regardless of the rate of subsequent warming. But when the cells were cooled to −30° or −75°, the results were different in two respects: survival

TABLE I

EFFECT OF COOLING RATE, MINIMUM TEMPERATURE, AND WARMING RATE ON THE SURVIVAL OF CELLS OF *SACCHAROMYCES CEREVISIAE*

Minimum temperature	Time at minimum temperature	Cooling rate*	Warming rate‡	No. of tubes	Plate count survival
°C	*min.*	*°C/min.*	*°C/min.*		*per cent*
−10	61	§	Slow (0.7)	2	80.9 ±1.3
	76		Rapid (400)	3	82.1 ±9.8
−30	45	Rapid (50)	Slow (0.9)	3	0.007±0.0005
	78		Rapid (630)	3	1.05 ±0.18
	10	Slow (1)	Slow (0.9)	2	4.11 ±1.45
	33		Rapid (630)	3	13.2 ±1.58
−75	72	Rapid (280)	Slow (0.9)	2	0.004±0.004
	152		Rapid (1100)	2	0.22 ±0.02
	10	Slow (1.4)	Slow (0.9)	2	0.93 ±0.15
	90		Rapid (1100)	2	16.7 ±5.9

* Cooling rate determined from −5° to 5° above minimum temperature. It does not include the time taken for the freezing of the external water.

‡ Warming rate calculated from the minimum temperature to 0°. It does not include the time taken for the thawing of the external ice.

§ Undetermined. Tubes immersed directly in bath at −10°; suspensions supercooled and were seeded to induce freezing.

dropped sharply, and at the same time became very sensitive to differences in the cooling and warming velocity. Slow *cooling* yielded 13 to 570 times as many viable cells as did rapid cooling. Slow *warming,* on the other hand, was detrimental to the cells and resulted in survivals of only 1/3 to 1/140 of those obtained with rapid warming. Moreover, the effects of rate of cooling and warming were additive in the sense that the combination of rapid cooling and slow warming was by far the most harmful of the four combinations and gave only 1/2000 to 1/4000 as many viable cells as did the least harmful combination of slow cooling and rapid warming.

Effect of Minimum Exposure Temperature. Several experiments were performed to determine in more detail the relation between the temperature to

which the cells were cooled and the percentage of survival. In all these experiments cooling was rapid and warming slow, the most harmful combination. As shown in Table II, survival decreased sharply between −10° and −30°. That the decrease

TABLE II

SURVIVAL OF CELLS OF *SACCHAROMYCES CEREVISIAE* AFTER BEING COOLED TO INDICATED TEMPERATURES AND WARMED SLOWLY

Two tubes used for each case

Exp. Number	Minimum temperature	Time at minimum temperature	Cooling rate	Warming rate	Cells unstained with methylene blue		Plate count survival
					5–7 hrs*	18 hrs*	
	°C	*min.*	*°C/min.*	*°C/min.*	*per cent*	*per cent*	*per cent*
1	−15	55	15°	0.65	57.8±5.0	50.2±7.3	37.6 ±5.8
	−30	40	50	0.75	6.2±2.6	2.0±1.1	0.0091±0.0004
	−45	23	50	0.80	7.7±1.2	3.2±0.6	0.0013±0.0002
	−60	14	50	0.90	7.2±0.2	3.3±0.2	0.0007±0.0002
	−75	10	50	1.0	6.1±2.5	2.0±0.5	0.0004±0.00007
	Control	—	—	—	98.0±0.4	97.9±0.4	100
2	−10	30	‡	0.55	80.3±1.4	78.7±0.3	73.2 ±3.7
	−15	23	17	0.61	31.0±1.3	24.5±2.5	23.4 ±3.4
	−20	17	17	0.68	14.2±0.0	11.4±0.3	10.0 ±0.0
	−25	13	17	0.75	9.6±6.2	7.9±6.2	4.1 ±3.5
	−30	10	17	0.82	10.2±2.7	5.1±2.8	2.2 ±1.9
	Control	—	—	—	97.9±0.2	98.6±0.6	100

* Interval between thawing and staining.

‡ Cooling rate is ambiguous since suspensions supercooled to −10°, warmed to 0° upon seeding, and then dropped again to −10° after completion of freezing.

was a direct function of temperature and not of variation in cooling rate is shown by Experiment 2 of Table II. There, cells were cooled to −15°, −20°, −25°, and −30° at the same average rate of 17°/min.; yet, 23 per cent survived exposure to −15° whereas only 2.2 per cent survived cooling to −30°. Nor could differences in the rate of warming have accounted for the temperature effects observed, for warming from the lower, more harmful temperatures was actually more rapid (and if anything, should have been protective) than warming from the higher temperatures.

Fewer cells survived cooling to −30° in Experiment 1 than in Experiment 2. This difference was probably a result of the higher cooling rate in the former.

Percentage survivals estimated from plate counts and the percentage of cells remaining unstained with methylene blue were qualitatively similar in that both dropped sharply between −10° and −30°; however, the percentage of unstained cells was somewhat the higher, a discrepancy that became large when only a small fraction of the cells survived. The fraction of cells unstained by methylene blue

decreased as the time between thawing of the suspensions and staining was lengthened from 7 to 18 hours, but remained appreciably higher than the percentage survival.

Several investigators have reported decreasing survivals of yeast with decreasing temperatures, (7-9) but the decrease was neither so extensive nor so abrupt as that found here.

Effect of the State of Extracellular Water. The abrupt decrease in survival below —10° was observed only when the water surrounding the cells was frozen. Thus, as shown in Table III, the percentage survival of cells in frozen water dropped sharply betwen —10° and —16°, whereas that of cells in supercooled water showed little or no drop. Actually, a slight decrease in survival seems apparent in cells in supercooled water at —16°, a decrease that may be real but on the

TABLE III

PERCENTAGE OF YEAST CELLS SURVIVING RAPID COOLING TO VARIOUS SUBZERO TEMPERATURES AND SUBSEQUENT SLOW WARMING WITH THE EXTRACELLULAR WATER EITHER SUPERCOOLED OF FROZEN

Tubes were immersed for a minimum of 10 minutes in ethanol baths previously cooled to temperatures indicated. Freezing above —10° was induced by seeding. Freezing below —10° was spontaneous. Freezing at —10° was of both sorts.

Exp. Number	Minimum temperature	Cell response			
		External water supercooled		External water frozen	
		Unstained with methylene blue	Plate count survival	Unstained with methylene blue	Plate count survival
	°C	*per cent*	*per cent*	*per cent*	*per cent*
1	−2	97.1±0.6(2)*	98.1±3.1(2)*	96.5±0.7(2)	93.3±5.6(2)
	−5	95.7±0.3(2)	106.0±1.0(2)	93.6±0.5(2)	97.0±2.6(2)
	−10	97.6±0.8(2)	103.2±0.5(2)	85.7±6.2(2)	77.6±0.15(2)
	−14	—	—	59.8±9.2(4)	61.0±11.5(4)
2	−15	98.2±0.8‡(1)	97.2±4.9‡(1)	54.2±0.8‡(1)	27.4±1.0‡(1)
3	−14	—	108.2±2.0(2)	—	40.8±3.4(4)
4	−5	—	93.8±2.5(2)	—	101.9±3.0(3)
	−10	—	101.4±3.4(3)	—	74.6±2.8(2)
	−12	—	103.4±3.7(3)	—	59.2±1.2(2)
	−13	—	96.8±3.4(3)	—	51.3±9.4(3)
	−14	—	104.8±11.5(3)	—	29.9±3.3(3)
	−15	—	89.4±2.8(2)	—	24.2±1.4(3)
	−16	—	84.4±5.3‡(1)	—	17.8±1.8(3)

* Numbers in parentheses refer to number of samples or tubes.

‡ Standard error based on the variance of the 3, 4, or 6 plates or 2 methylene blue slides prepared from the single sample available. In all other cases it is calculated from the individual mean survivals of the 2 or 3 samples used for each treatment.

other hand may be merely a statistical artifact arising from the fact that only one sample (of 17) remained supercooled at that temperature. The matter could be decided if it were possible to supercool suspensions to still lower temperatures. Unfortunately, the chances of successfully doing so with the procedures used seem slim, since the one instance of supercooling to —16° could not be repeated.

A plot of survival as a function of temperature (Fig. 1) shows the abruptness

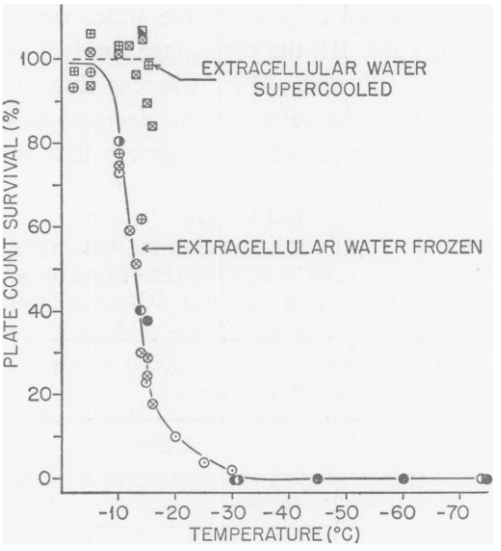

FIGURE 1 Percentage of yeast cells in frozen or supercooled water surviving rapid cooling to various temperatures and subsequent slow warming. All datum points represented by a given symbol represent results from the same experiment. Circles—extracellular water frozen; squares—extracellular water supercooled.

with which survival plummets between —10° and —20° when cells are in frozen water and the lack of injury when they are in supercooled water.

The injurious effects of low temperature exposure occurred extremely rapidly, for when cells were rapidly cooled to —30°, an exposure of 1 minute lowered viability from 100 per cent to 1 per cent (Table IV). Longer exposures, up to 90 minutes, produced little if any further decrease.

Effect of Degree of Supercooling on Cooling Velocity and Survival. It has already been noted (Table I) that suspensions cooled slowly at 1°/min. yielded appreciably higher survivals than those cooled at higher velocities. Actually, the cooling rate of 1°/min. refers to the rate of decrease in the bath temperature. Although it was constant, the cooling rate of the suspensions was not. As indicated in Fig. 2, the spontaneous termination of supercooling resulted in an abrupt rise in the temperature of the suspension to 0° followed shortly thereafter (upon the complete freezing of all water) by rather rapid cooling back down to the temperature

TABLE IV

SURVIVAL OF YEAST CELLS AS A FUNCTION OF TIME AT −30°

Cooling and warming to and from −30° were rapid.

Time at −30°	No. of tubes	Plate count survival
min.		*per cent*
0*	3	100*
1	3	1.54±0.26
3	3	1.25±0.08
10	3	0.69±0.005
30	3	0.73±0.10
90	3	0.94±0.06

* Unfrozen control; survival 100 per cent by definition.

of the bath. In the particular example shown, the cooling after the completion of freezing was at the rate of 13°/min. even though the rate of fall of the bath temperature was 1°/min.

Since this period of more rapid cooling could have occurred just as the cells were entering the harmful temperature zone (< −10°), it might have been damaging to the cells. Or to put the matter differently, "slow" cooling might have been even more protective to the cells if this short period of rather rapid cooling had been avoided. The best way to avoid it would be to seed the suspensions just below 0°, say at −2°, allow them to solidify at that temperature, and then permit the temperature of the cooling bath to drop slowly. When this was done, as shown in Table V, 35 per cent of the cells survived cooling to −30° and subsequent slow warming. On the other hand, when the suspensions supercooled to −16° before freezing spontaneously during the course of a slow lowering in bath temperature, only 14 per cent of the cells survived.

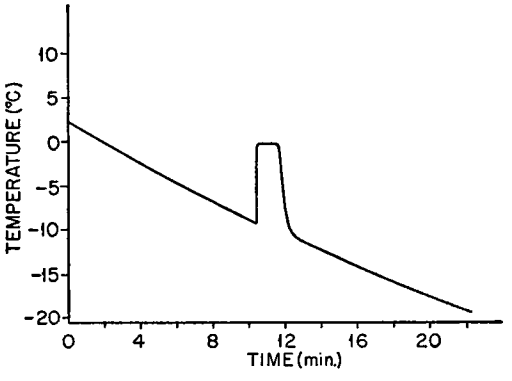

FIGURE 2 Cooling curve recorded for 0.1 ml of distilled water in a freezing tube immersed in an ethanol bath cooling at 1°/min.

TABLE V

RELATION BETWEEN THE TEMPERATURE AT WHICH SUPERCOOLING WAS TERMINATED AND THE SURVIVAL OF YEAST CELLS ULTIMATELY COOLED TO −30° AND WARMED SLOWLY

Cooling rate of bath	Temperature at which freezing occurred	Method of freezing	No. of tubes	Plate count survival
°C/min.	*°C*			*per cent*
1	−2.5	Seeded	3	34.6 ±3.0
1	−16	Spontaneous	2	13.9 ±1.3
50*	Not observed	Spontaneous	3	0.0033±0.0007

* Rate of cooling of suspension from −5° to −25°. Tube immersed directly in bath at −30°.

The precaution of eliminating the spurt of fast cooling introduced by supercooling thus enhances the contrast between the protective action of true slow cooling at 1°/min. and the destructive action of rapid cooling at 50°/min. Some 35 per cent survived in the former case, but only 0.003 per cent in the latter.

Survival of Yeast Cells in Frozen Aqueous Solutions. Unlike distilled water, which was the suspending medium in the experiments described to this point, aqueous solutions exhibit progressive ice formation with decreasing temperature, a process that culminates at the eutectic point, the temperature below which no liquid remains. Several experiments were performed involving the exposure to subzero temperatures of yeast cells suspended in solutions of various solutes to see if sur-

TABLE VI

PERCENTAGE OF YEAST CELLS SURVIVING EXPOSURE TO −30° FOR 10 MINUTES WHILE SUSPENDED IN VARIOUS AQUEOUS SOLUTIONS

Exp. No.	Suspending vehicle	No. tubes/ treatment	Viable cell count (× 10^6/ml)		Plate count survival in frozen suspensions*
			Unfrozen control	Frozen suspension	
					per cent
1	Dist. H_2O	2	102.0	2.25	2.21±0.22
Rapid cooling,‡ rapid warming	0.1M KH_2PO_4	2	109.5	0.424	0.39±0.08
	0.1M NaCl	2	117.5	17.0	14.5 ±1.0
	0.1M $CaCl_2$	2	112.3	4.30	3.83±0.41
	Growth medium	2	118.8	7.41	6.24±0.40
2	Dist. H_2O	3	105.5	65.0	61.5 ±2.5
Slow cooling,§ slow warming	0.1M KH_2PO_4	3	88.6	21.7	24.5 ±3.7
	0.1M NaCl	3	128.4	67.7	52.8 ±1.1
	0.1M $CaCl_2$	3	141.8	37.7	26.6 ±1.0

* Based on the viable cell count in the corresponding control solution.

‡ Tubes immersed directly in bath at −30°; suspensions cooled at about 50°/min.

§ All suspensions seeded at −5° before allowing bath to cool at 1°/min. to −30°.

vival would be affected by the progressive freezing or by the location of the eutectic point. The solutions used were 0.1 M concentrations of KH_2PO_4, NaCl, and $CaCl_2$ with eutectic points of —2.7°, —21.1°, and —50°, respectively (10, 11). The standard growth medium (see Methods) was used as an additional suspending medium in one experiment, and samples of cells in deionized distilled water were also treated. The percentages of cells surviving the sequences of (*a*) rapid cooling to —30° with subsequent rapid warming and (*b*) slow cooling to —30° with subsequent slow warming are summarized in Table VI. Several aspects of these data are of interest. In the first place, the presence of inorganic solutes in the extracellular fluid was not in itself detrimental to the cells; in fact, 0.1 M NaCl seemed to protect the cells during rapid cooling. Second, as was the case for cells suspended in distilled water, slow cooling was considerably less injurious than rapid cooling. Finally, there seemed to be no particular correlation between survival in a particular solution and whether the minimum exposure temperature of —30° was above or below the eutectic point of the solution.

The survival of yeast in solutions of 0.1 M KH_2PO_4, NaCl, and $CaCl_2$ was studied further by cooling suspensions to several temperatures between —2° and —76° and warming them slowly. The resulting percentage survivals as a function of temperature are plotted in Fig. 3. In all these solutions, as in the case of distilled water

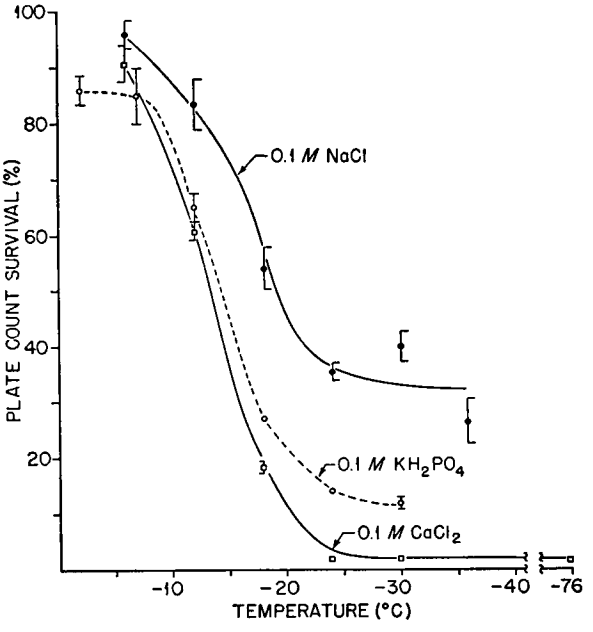

FIGURE 3 Percentages of yeast cells surviving cooling to various subzero temperatures while suspended in 0.1 M solutions of NaCl, KH_2PO_4, or $CaCl_2$. Cooling was carried out stepwise by transferring cooling tubes successively to baths held at the indicated temperatures, the cooling velocity being 8 to 15°/min. Suspensions at —2°, and —6°, and half of those at —12° were seeded to induce freezing; the others froze spontaneously. Warming was slow.

(Fig. 1), there was a sharp drop in survival between —10° and —30°. It should be noted that the temperature range in which this drop occurred was independent of the eutectic points of the external solutions, for as mentioned, these eutectic points ranged from —2.7° for KH_2PO_4 to —50° for $CaCl_2$. The percentage of cells surviving exposure to the lower temperatures did, however, differ somewhat in the various solutions. These differences may be reflections of the response of the cells to the particular solutes, but they may reflect other unknown and uncontrolled variables since the experiments were carried out on different days with different populations of cells. Nevertheless, the higher survivals in the presence of 0.1 M NaCl noted in Table VI were also apparent in the experiments summarized in Fig. 3.

Solidification Temperatures of the Suspending Fluids. Theoretically, complete solidification of the 0.1 M solutions of NaCl, KH_2PO_4, and $CaCl_2$ used in the experiments already described should have occurred at the eutectic points of these solutions. However, Rey (12) has found that in practice such solutions must be cooled a number of degrees below that point before complete freezing is attained.

Measurements of the resistivity of these solutions as a function of temperature (Fig. 4) support Rey's findings. Complete solidification, as evidenced by a resistivity of 10^6 ohm-cm or more, occurred at —12°, —30°, and —71° for KH_2PO_4 (eutectic —2.7°), NaCl (eutectic —21.1°), and $CaCl_2$ (eutectic —50°). On the

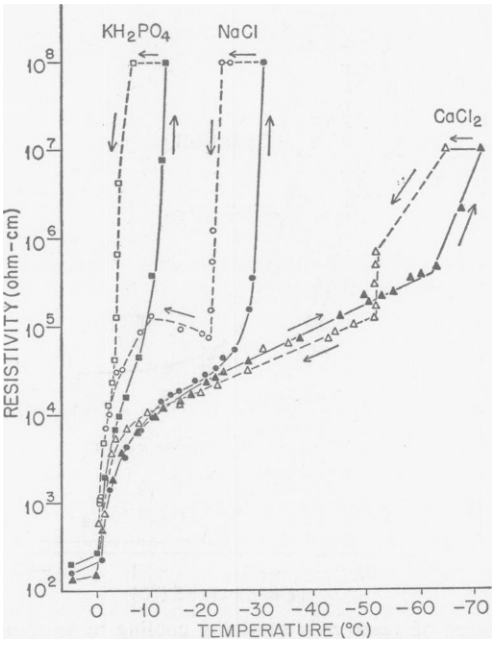

FIGURE 4 Electrical resistivity of 0.1 M solutions of KH_2PO_4, NaCl, and $CaCl_2$ as a function of temperature. Measurements were made during cooling (solid lines) and during warming (broken lines).

other hand, during warming the first signs of melting were observed near the true eutectic points. This is indicated by the initial steep drop in resistivity in the plots of Fig. 4, occurring at —4°, —22°, and —52° for the three solutions, respectively. The rate of warming of the solutions decreased at these same temperatures, confirming the occurrence of an endothermic phenomenon, *i.e.*, melting.

The presence of 2×10^8 yeast cells/ml in the solutions did not alter the relation between resistivity and temperature significantly (Fig. 5). The same cooling-warm-

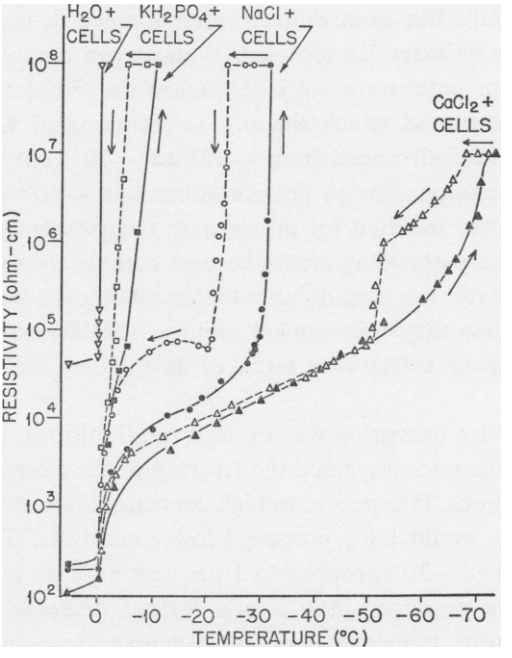

FIGURE 5 Electrical resistivity of a suspension of yeast cells in deionized distilled water or in 0.1 M solutions of KH_2PO_4, NaCl, or $CaCl_2$ as a function of temperature. Measurements were made during cooling (solid lines) and during warming (broken lines).

ing hysteresis was observed with complete solidification occurring at —11°, —30°, and —71° for KH_2PO_4, NaCl, and $CaCl_2$, respectively.

The electrical resistivity of a suspension of cells in deionized distilled water was 3.5×10^4 ohm-cm at 25°, equal to the measured resistance of a 0.0002 M NaCl solution; therefore, these cells were actually suspended in a very dilute aqueous solution, which might require temperatures below 0° to attain complete solidification. But an experiment showed that solidification was complete at —0.3°, for the resistivity became greater than 10^7 ohm-cm at that temperature.

As already noted, the drop in survival of the yeast occurred primarily between —10° and —30°, and occurred, therefore, below the solidification temperature of

distilled water and KH_2PO_4 and above the solidification temperature of NaCl and $CaCl_2$.

DISCUSSION

Death of cells at subzero temperatures must be a result either of low temperatures *per se* or of the freezing of water. In yeast cells, low temperatures were not injurious to supercooled suspensions; freezing of the water surrounding the cells was required for death. But even though external freezing was a prerequisite for death, it was not the primary lethal agent. Water froze completely above —0.3°; yet, cells suspended in water were not killed unless the temperature dropped below —10°. The temperatures at which the 0.1 M solutions of KH_2PO_4, NaCl, and $CaCl_2$ completely solidified ranged from —12° to —70°, but the survival of cells suspended in these solutions always decreased between —10° and —30°. In other words, the temperatures required for injury were completely unrelated to the temperatures at which the suspending media became completely solidified.

Furthermore, survival was considerably higher when cells were cooled slowly to below —10° than when they were cooled rapidly. This dependency of survival on cooling velocity seems inexplicable in terms of death being a direct consequence of external freezing.

Since extracellular ice formation was in itself non-injurious, the progressive concentration of solutes that accompanied the freezing of the electrolyte solutions must also have been innocuous. If exposure to high concentrations of salt had been harmful, longer exposures would have produced lower survivals. They did not do so. The survival of yeast at —30° dropped to 1 per cent after an exposure of 1 minute and longer exposures produced little if any further decrease. Slow cooling produced a longer exposure than rapid cooling; yet many more cells survived in the former case even when suspended in 0.1 M salt solutions, the solute concentrations of which were unequivocally rising during that cooling.

The high concentration of extracellular solutes produced by freezing (particularly electrolytes) has been held by many investigators to be one of the important causes of death at low temperatures (*cf.* the work of Lovelock (13), Lovelock and Polge (14), and reviews by Smith (15), Meryman (16), and Rey (17)). The general applicability of this hypothesis is questionable. It seems inapplicable to yeast and to at least two other species of microorganisms (5), and appears to be of uncertain validity with respect to mammalian red blood cells (18) and bone marrow cells (19).

An alternative hypothesis is that death in yeast was due not to extracellular freezing but to intracellular freezing. The abruptness with which survival dropped with respect to both temperature and time supports the suggestion that a change in state was involved. Intracellular freezing would produce a progressive increase in the

concentration of intracellular solutes, an increase that conceivably could cause death. However, the same evidence that dictated against the idea of death from concentrated extracellular solutes would also rule against death from concentrated intracellular solutes; namely, longer exposures to decreasing temperatures were not more harmful than shorter exposures. In fact, longer exposures were less harmful as witnessed by the protective effect of slow cooling.

We suggest, therefore, that death of the yeast occurred because of the physical-chemical consequences of the presence of ice crystals within the cell, and not because of the removal of liquid water by its conversion to ice. Indeed, the removal of intracellular water seems to be the mechanism by which slow cooling reduced the lethality of low temperature exposure. If during cooling, the external medium begins to freeze while the cell is still supercooled, the vapor pressure within the cell would be higher than that of the ice or solution outside the cell. Because of this vapor pressure differential, water would tend to flow out of the cell and freeze externally. Slow cooling would provide more time for this outflow and would produce more extensive dehydration than would rapid cooling. Conceivably, dehydration could be sufficient to withdraw all the water of normal activity, leaving behind only intracellular water with activity sufficiently reduced to render it incapable of freezing. These theoretical consequences of a low cooling velocity are supported by observations on yeast (20, 21), cells of higher plants (22), and certain animal cells (23) by various investigators. What they observe under the microscope is that intracellular and extracellular ice crystals form when cooling is rapid. When it is slow, freezing is extracellular only. In the latter case, water apparently is withdrawn from the cells, as evidenced by their collapse (yeast or animal cells) or plasmolysis (cells of higher plants).

If death is caused by intracellular freezing, as postulated, the finding that survival after exposure to low temperatures was not markedly affected by the presence of salts in the external medium is not unexpected. The vapor pressure differential, and hence the tendency for water to flow out of the cells, would be the same at a given subzero temperature regardless of whether the external medium was completely frozen pure water or an incompletely frozen aqueous solution if no solutes permeated the cell. However, there could be specific differences in the effect of individual ions on survival, depending on such factors as the relative permeability of the yeast membrane to specific ions (24) or the effect of specific ions on the structure or composition of the plasma membrane (25), and such factors may have been responsible for the differences in the level of survival noted in the various solutions.

The suggestion that intracellular freezing is the lethal factor is faced by one challenge, and that is to account for the finding that injury did not occur in the absence of *extra*cellular freezing and did not occur in the presence of extracellular freezing unless the temperature dropped to $-10°$ or below. To state the matter differently,

why should the occurrence of intracellular freezing require the preexistence of extracellular ice and temperatures below −10°?

The easiest explanation would be simply that extracellular ice seeds supercooled intracellular water and thus initiates the freezing of the latter. The difficulty with this explanation is twofold: first of all, Chambers and Hale (26) and Luyet and Gibbs (27), among others, have observed that the cell membranes of amebae and of muscle and onion cells act as a barrier to prevent seeding; second, in the present case of yeast, why would not such seeding have occurred above −10°? A possible but admittedly speculative solution to these difficulties has been suggested elsewhere (5). In brief, the proposal is that extracellular ice seeds intracellular water by growing through the water-filled pores or channels in the cell wall or cell membrane. Whatever their nature, the size of these channels based on permeability studies must certainly be small—on the order of 10 to 30 A in radius (28)—and to pass through a pore of such size, an ice crystal must have a radius of curvature less than the radius of the pore. According to physical theory, however, crystals of such radii cannot exist at 0° but only at lower temperatures, and the smaller the radius, the lower the temperature required for existence and growth of the crystal (29-31). The speculation, then, is that the cell wall or cell membrane is a barrier to seeding at temperatures near 0° but ceases to be a barrier when the temperature drops low enough to permit the existence and growth of crystals small enough to pass through the water-filled pores. The exact temperature at which this would occur in an individual cell would depend on the radius of the larger pores in that particular cell. Some support for this proposal is found in Lusena and Cook's studies with model membranes (32) and in Asahina's observations on plant cells (22) that semipermeable membranes can in fact be barriers to seeding at higher temperatures and cease to be barriers at lower temperatures. The curvature of water surfaces is also believed by Salt to be one factor affecting the ability of insects to supercool to low temperatures (33).

The validity of the speculation hinges on the correctness of the inferred causal relation between intracellular freezing and survival in yeast. The inference is supported by Nei's direct microscopic observations on yeast cells exposed to low temperatures (20). He found that cell suspensions seeded above −10° showed high survivals (53 to 80 per cent unstained by methylene blue) and only extracellular ice. Cell suspensions frozen after supercooling to −20° and those frozen by rapid cooling to −78° or −190° showed low survivals (5 to 18 per cent) and intracellular ice crystals.

One factor not yet brought into the discussion is the effect on survival of the rate at which frozen yeast suspensions were warmed; namely, the finding that rapid warming yielded higher survivals than did slow warming, also reported by Lund and Lundberg (34). Although rate of warming exerted considerable influence on survival, especially when the other factors of temperature and cooling rate were such

as to be highly deleterious, it was not the factor of paramount importance. This situation is somewhat different from that found with *Aspergillus* spores (3) and cells of *Pasteurella tularensis* (6), in which rate of warming appeared to be the final determiner of whether survival would be high or low.

The deleterious effects of rapid cooling and slow warming may possibly be related to the fact that the size of ice crystals is partly determined by the rate at which the latent heat of fusion is withdrawn from the system. Since the rapid withdrawal of heat yields small crystals, the internal ice crystals in rapidly cooled cells should be small. Small crystals, however, have more free energy (higher vapor pressure) than larger ones, so that during warming there will be a tendency for the larger to grow at the expense of the small, a process known as recrystallization. Hence, slow warming may be more harmful than rapid because it allows more time for the ice crystals to enlarge to a lethal size before melting, a possibility that has been discussed elsewhere in more detail (5).

The plate-count technique equates survival with cell reproduction, but the rough equivalence found between survivals based on colony counts and the percentage of cells unstained by methylene blue suggests that death involved considerably more than loss in ability to reproduce. Indeed various investigators have found other manifestations of low temperature injury in yeast including permeability changes (35, 36), decrease in respiration and fermentation and changes in enzymatic activities (37-41), and structural changes (39, 42). Intracellular ice formation may be responsible for all these alterations; but even if it is, there still remains the question of the mechanisms involved in the translation of this physical event into cell injury.

The technical assistance of Miss Ella Jane Holloway in the latter phases of this investigation is gratefully acknowledged.

Preliminary reports of portions of this work have been presented elsewhere (4, 5).
The earlier portion of the study was performed at Princeton University under the auspices of a National Science Foundation Postdoctoral Fellowship and supported in part by the Whitehall Foundation, Inc., New York. It was aided by suggestions and advice from Professor A. K. Parpart.
The Oak Ridge National Laboratory is operated by the Union Carbide Corporation for the United States Atomic Energy Commission.
Received for publication, September 12, 1960.

REFERENCES

1. FERNÁNDEZ-MORÁN, H., *Ann. New York Acad. Sc.*, 1960, **85**, 689.
2. PARKES, A. S., and SMITH, A. U., (editors), Recent Research in Freezing and Drying, Oxford, Blackwell Scientific Publications, 1960.
3. MAZUR, P., *J. Gen. Physiol.*, 1956, **39**, 869.
4. MAZUR, P., *in* Recent Research in Freezing and Drying (A. S. Parkes and A. U. Smith, editors), Oxford, Blackwell Scientific Publications, 1960, 65.

5. MAZUR, P., *Ann. New York Acad. Sc.*, 1960, **85**, 610.
6. MAZUR, P., RHIAN, M. A., and MAHLANDT, B. G., *Arch. Biochem. and Biophysics*, 1957, **71**, 31.
7. BAUM, H. M., *Biodynamica*, 1943, **4**, 71.
8. CAMPBELL, J. D., *Biodynamica*, 1943, **4**, 65.
9. WOOD, T. H., and ROSENBERG, A. M., *Biochim. et Biophysica Acta*, 1957, **25**, 78.
10. VAN DEN BERG, L., and ROSE, D., *Arch. Biochem. and Biophysics*, 1959, **81**, 319.
11. SEIDELL, A., and LINKE, W. F., Solubilities of Inorganic and Organic Compounds, New York, suppl. 3rd edition, D. Van Nostrand Co., Inc., 1952.
12. REY, L. R., *Ann. New York Acad. Sc.*, 1960, **85**, 510.
13. LOVELOCK, J. E., *Biochim. et Biophysica Acta*, 1953, **10**, 414.
14. LOVELOCK, J. E., and POLGE, C., *Biochem. J.*, 1954, **58**, 618.
15. SMITH, A. U., *in* Biological Applications of Freezing and Drying (R. J. C. Harris, editor), New York, Academic Press, Inc., 1954, 1.
16. MERYMAN, H. T., *Science*, 1956, **124**, 515.
17. REY, L. R., *Experientia*, 1959, **15**, 449.
18. RINFRET, A. P., *Ann. New York Acad. Sc.*, 1960, **85**, 576.
19. PHAN THE TRAN and BENDER, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1960, **104**, 388.
20. NEI, T., *J. Agric. Chem. Soc. Japan*, 1954, **28**, 91.
21. NEI, T., *in* Recent Research in Freezing and Drying (A. S. Parkes and A. U. Smith, editors), Oxford, Blackwell Scientific Publications, 1960, 78.
22. ASAHINA, E., The Freezing Process of Plant Cell, *Contrib. Inst. Low Temp. Sc.*, Hokkaido University, Sapporo, Japan, No. 10, 1956, 83.
23. MERYMAN, H. T., and PLATT, W. T., Naval Medical Research Institute Research Report Project NM 000 018.01.08, 1955.
24. ROTHSTEIN, A., *Bact. Rev.*, 1959, **23**, 175.
25. LOVELOCK, J. E., *Nature*, 1954, **173**, 659.
26. CHAMBERS, R., and HALE, H. P., *Proc. Roy. Soc. London, Series B*, 1932, **110**, 336.
27. LUYET, B. J., and GIBBS, M. C., *Biodynamica*, 1937, **1** (25), 1.
28. MITCHELL, P., *Ann. Rev. Microbiol.*, 1959, **13**, 407.
29. CHALMERS, B., *Scient. Am.*, 1959, **200** (2), 114.
30. STEPHENSON, J. L., *in* Recent Research in Freezing and Drying (A. S. Parkes and A. U. Smith, editors), Oxford, Blackwell Scientific Publications, 1960, 121.
31. STEPHENSON, J. L., *Ann. New York Acad. Sc.*, 1960, **85**, 535.
32. LUSENA, C. V., and COOK, W. H., *Arch. Biochem. and Biophysics*, 1953, **46**, 232.
33. SALT, R. W., *J. Insect Physiol.*, 1958, **2**, 178.
34. LUND, A. J., and LUNDBERG, W. O., *Ann. Rep. Hormel Inst., Univ. Minnesota*, 1949-50, 47.
35. FOULKES, E. C., *J. Bact.*, 1954, **68**, 505.
36. CONWAY, E. J., and DUGGAN, F., *Biochem. J.*, 1958, **69**, 265.
37. OGAWA, T., *Teion Kagaku* (*Low Temp. Sc.*), 1953, **10**, 175.
38. NEI, T., OGAWA, T., KANEHIRA, S., and AKIMOTO, H., *J. Agric. Chem. Soc. Japan*, 1954, **28**, 94.
39. HANSEN, I. A., and NOSSAL, P. M., *Biochim. et Biophysica Acta*, 1955, **16**, 502.
40. SAKAGAMI, Y., *Teion Kagaku* (*Low Temp. Sc.*), 1959, Series B, **17**, 105.
41. CHRISTOPHERSEN, J., and PRECHT, H., *Biol. Zentr.*, 1956, **75**, 612.
42. NEI, T., SAKAUSHI, E., SAKAGAMI, Y., and SATO, S., *Teion Kagaku* (*Low Temp. Sc.*), 1959, Series B, **17**, 63.